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OBSERVATIONS ON THE METABOLISM OF *SARCINA LUTEA*. I

R. W. GERARD AND I. S. FALK

(From the Departments of Physiology and of Hygiene and Bacteriology, University of Chicago)

The experiments reported in this communication were designed to provide some information on the dynamics of oxygen diffusion into living cells. It seemed desirable, *a priori*, to perform the observations on very small, spherical cells. To this end, micrococci were the forms chosen. After preliminary experimentation, it was found that a strain of *Sarcina lutea* which had long been maintained on artificial culture media fulfilled many of the requirements of the experiments: the organisms are very small and discrete (this strain no longer forms packets); the cells are spherical and highly uniform in diameter; and in water or dilute aqueous solutions they remain sufficiently viable for the requisite periods of time under the conditions used. As the experiments progressed there were noted a number of interesting characteristics of their metabolism, which are the main subjects of this paper.

Methods

1. *Bacterial Cultures.*—(*a*) Preparation of suspensions: The organisms grow luxuriantly on plain nutrient agar prepared with peptone and fresh meat infusion or beef extract, with or without the addition of NaCl. After the completion of the preliminary experiments, the medium used contained one per cent Difco peptone, 0.35 per cent beef extract and 1.5 per cent agar in tap water. Agar slant cultures in tubes or Kolle flasks were grown for 20–24 hours at 37° C. The growth was washed off with distilled water, the suspension homogenized by gentle but prolonged shaking, the microörganisms precipitated by centrifugation and then washed by two suspensions in and

precipitation from distilled water. After the final washing, the sedimented cells were again suspended in distilled water and shaken to give a concentrated homogeneous suspension.

(*b*) Enumeration of cells and pH measurements: The numbers of bacteria per cc. were determined before and after exposure to the specific conditions of the experiments by direct microscopical observation and by plate count. The former was performed by the hemocytometer method, using a dilute solution of carbol fuchsin as a diluting fluid; the latter as commonly used in the enumeration of bacteria in water, milk and other fluids. The direct count includes all cells visible under the microscope and the plate count only viable cells which developed on the medium used into colonies visible to the eye aided by a 5–10 × hand lens. Although all reasonable efforts were made to insure accuracy, the usual presumptive errors of these methods apply to our data.

The suspensions used in our experiments were, before dilution, thick, yellow, pasty emulsions of the sedimented, packed cells. Microscopical observations indicate a high degree of freedom from detritus. The concordance of the direct and plate counts made before and after performance of the experiments implies a high coefficient of vitality and the essential absence of dead or senile cells in the suspensions when first prepared.

Measurements of hydrogen ion concentration were performed with indicators by a spot plate method. Inasmuch as the suspensions consist of the bacterial cells in distilled water or dilute aqueous solutions of low intrinsic buffering power, the stability of the pH of an exposed suspension is controlled primarily by the buffering powers of the cells. From preliminary titration experiments (after the method of Shaughnessy and Falk, 1924) it developed that the strain of *Sarcina lutea* used displayed rather slight buffering power. Hence, in performing colorimetric pH measurements, we determined with which of a series of solutions of very low buffer action, and varying but known pH values, the test solution was isohydric. The standard solutions were prepared by adding twice the usual concentration of indicator solution to water and adjusting the pH of 10 cc. samples to various levels (at 0.2 pH intervals) by adding appropriate quantities of HCl or NaOH (*cf.* Fawcett and Acree, 1929). The colorimetric standards were checked by electrometric measurements, using a buffer solution standard and the quinhydrone electrode. The results of several experiments appear in Table I.

It will be noted that the direct and plate counts generally agree closely at the outset of an experiment and are not too widely apart at

TABLE I

Hydrogen Ion Concentrations and Microbic Enumerations for Suspensions of Sarcina Lutea

Date 1929	Suspension	pH	Number of cocci per cc. ($\times 10^{-3}$)	
			Direct	Plate
Apr. 27	Original		3.2	1.7
	After 2 hours (room temperature):			
	Original		2.6	2.1
May 30	Original	7.4	143	140
	After 6 hours:			
	In water shaken in air	6.8	107	430
June 6	Original	7.4	63	64
	After 6 hours:			
	Original	7.2	150	30
	In glucose shaken in air	?	150	30
	In water shaken in N_2	7.6	32	27
	In glucose shaken in N_2	7.5	41	27
	In water shaken in air	8.0	40	7
July 5	Original		70	26
	After 6 hours:			
	Original		102	29
	In water shaken in air		23	9
	In glucose shaken in air		27	11
	In glucose shaken in 1% O_2		13	14
	In water shaken in 1% O_2		58	13
Aug. 7	Original		85	13
	After 11 hours:			
	Original	7.4	71	17
	In water shaken in air	7.8	23	8
	In glucose shaken in air	7.2	8	6
	In water shaken in N_2	7.2	6	8
	In glucose shaken in N_2	6.4	14	6
Oct. 16	Original	7.4	199	55
	After 26 hours:			
	In water shaken in N_2	7.8	182	29
	In glucose shaken in N_2	6.4	202	180
	In glucose shaken in air	6.0	139	35
	In water shaken in N_2	7.8	170	35
	In water shaken in air	7.6	148	32

the completion. When variations occur they are generally, as is to be anticipated, in the direction of lower plate than direct counts.

(*c*) Size of the bacteria: The diameters of the bacterial cells were determined by direct microscopical measurements with hanging drop preparations and a filar micrometer. There were no significant variations in the size of cells suspended in water and in dilute glucose suspensions, with or without traces of dilute, neutral fuchsin. The average diameter determined from several hundred measurements on the cocci was 1.275 micra. In this series the maximum was 1.42, the minimum 1.08. The distribution was approximately symmetrical about the mean and the probable error of the mean was $\pm$ 0.07 μ.

(*d*) Final handling: The thick bacterial paste obtained after centrifugation was diluted 1 : 1 with distilled water or with 1.0 or 0.5 per cent glucose in water and well mixed by shaking (with a glass bead). Of these suspensions 0.4 cc. (dry weight about 5 mgm., containing about 2×10^9 bacteria) or 0.8 cc. portions were placed in the manometer vessels for determination of oxygen consumption, etc., and a similar amount dried on a cover slip and weighed. In certain experiments oxygen was bubbled through the suspension for some time before beginning the observations. The pH was usually determined before and after the manipulation by the method described above.

2. *Gases.*—Tank nitrogen, of 99.7 per cent purity, was passed over heated copper and through glass connections directly into the manometers. Even this treatment did not give absolute freedom of oxygen, but traces that remained did not affect the results (see below). Gas mixtures containing 0.5 to 10 per cent oxygen were made by volumetric mixing of the treated nitrogen and air in large bottles over water. The nitrogen was always bubbled through first for a considerable time to remove dissolved air, and the mixture was used at once after preparation. To obtain accurate mixtures of 0.5 per cent or less, a manometric method was used. This is described below.

3. *Measurement of Oxygen Consumption, Carbon Dioxide Production, etc.*—The Warburg methods were used throughout. Small chambers of the cylindrical or conical type with an inset, used for studying metabolism of nerve (Gerard, 1927), were found highly satisfactory. With 0.4 cc. of bacterial suspension and 0.2 cc. of $N/10$ NaOH in the inset to absorb CO_2, the constants were either about 0.25 or 0.35, so that one cu. mm. change in gas volume gave a pressure difference of 3 or 4 mm. (of Brodie's solution). Oxygen consumption, Q_{O_2}, is expressed as cu. mm. consumed per hour per mgm. dry weight of the bacteria.

The respiratory quotient (R.Q.) was determined by comparison of the manometer readings when CO_2 was absorbed (NaOH in inset) and when allowed to accumulate (water in inset) in parallel experiments. Acid production was determined manometrically by the liberation of CO_2 from a bicarbonate-Ringer solution in the presence of 5 per cent CO_2 in nitrogen (Warburg, 1926).

All experiments were carried out at 20.1° C.

Gas mixtures with low concentrations of oxygen were obtained by mixing in a manometer. The Brodie solution used in the manometer capillary has a specific gravity so adjusted that 10,000 mm. equals 760 mm. of mercury. The pressure of the gas in the manometer chamber is easily obtained from the barometer reading and the difference of fluid level in the two limbs of the manometer. It is usually sufficiently accurate, in determining the percentage of oxygen, to assume 10,000 mm. Even the maximal correction for water vapor, if dry air is admitted, is only 5 per cent and may ordinarily be omitted. The oxygen admitted to the chamber filled with nitrogen is determined as follows: the fluid levels in the manometer are lowered (by a screw at the bottom) until there is a "negative" pressure in the chamber of *circa* 100 mm. (left side open to air = 50 mm., right side to chamber = 150 mm.). The chamber is then opened to the air for an instant by rapidly turning a stop-cock, the apparatus is shaken for a moment, the fluid level on the chamber side is brought to its initial setting and the level in the open limb read. This will now be, say, 200 mm. At constant volume and temperature then (the room air being nearly at the temperature of the thermostat and coming to rapid equilibrium) the gas pressure has been increased by 150 mm. of air or 30 mm. of oxygen. With a little practice it is possible to adjust the initial pressure difference and time of opening the chamber so that any desired oxygen percentage up to 0.5 can be obtained by one operation. This method permits the rapid and accurate preparation of gas mixtures with very low oxygen concentration and should be valuable in other studies where the influence of oxygen pressure on oxygen consumption is to be determined.

Results

1. *Respiration in Air.*—The rate of oxygen consumption for any sample of the bacteria used is not constant with time, but tends to be high at first and to fall rapidly at the outset and then slowly. Sometimes it remains at a constant low level from the third or fourth hour on; more often a slow fall continues through a 20-hour period. A typical curve is shown in Fig. 1, and the results of several experiments are given in Table II.

The initial level of oxygen consumption and the amount of fall have been fairly constant from one test suspension to another and variations are not related to variations in the ages of the cultures used. It might be anticipated that bacteria taken from a culture which has passed the period of logarithmic rate of growth would show an approxi-

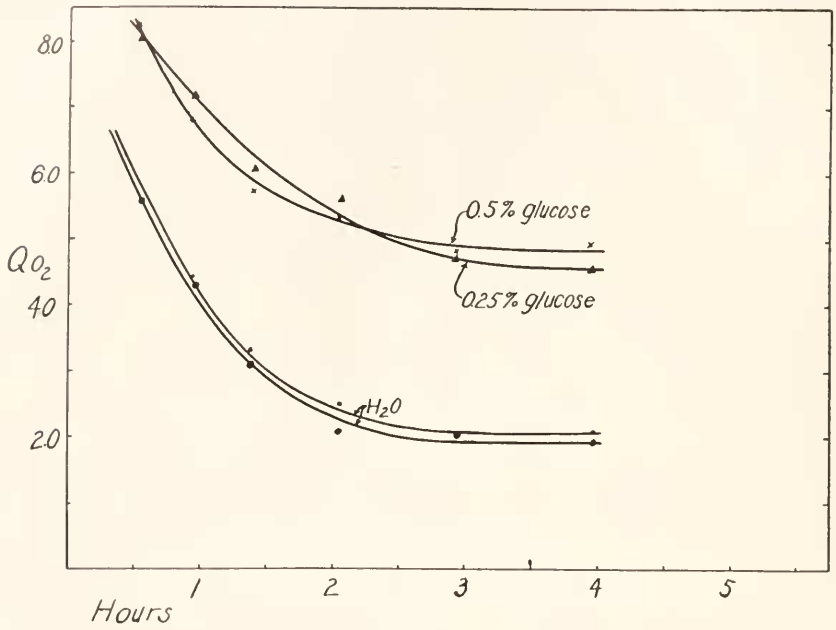

FIG. 1.

mately uniform level of oxygen consumption. Although viability generally decreases in the non-nutrient menstrua, the rate of this process is far too low to be invoked as the explanation of the decline in oxygen consumption. The question arises: In what way does the preparation of the suspension and its treatment in the manometer chamber lead to the establishment of a "time zero"? In other words, if the oxygen consumption of these cocci is assumed to have been essentially constant in time, what causes it to begin a rapid fall when its measurement is undertaken?

If a progressing mortality of the individual cells does not seem to explain the findings, the early high respiration cannot, on the same evidence (some variable fall in plate counts from start to end), be interpreted as due to continued growth which was later suspended. Continued agitation, gentle or vigorous, has been shown to have no effect; and temperature and suspension menstruum can also be

TABLE II

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
May 14	Water suspension, in air	6.0	3.5	2.3		1.6	
	Water suspension, in air	6.5	3.5	2.3		1.7	
	0.2% glucose suspension, in air		5.5	3.8		2.4	
	0.2% glucose suspension, in air		5.5	3.4		2.2	
May 30	Water suspension, in O_2	4.4	2.2		2.0		
	Water suspension, in air	4.3	2.0		2.0		
	0.5% glucose suspension, in air	6.9	5.1		5.0		
	0.2% glucose suspension, in air	7.1	5.2		4.6		
June 6	Water suspension, in air		6.8	5.2	4.0	3.3	2.8
	Water suspension, in 1% O_2		2.9	2.3	2.0	1.8	1.6
	0.2% glucose suspension, in 1% O_2		5.0	3.7	3.0	2.6	2.3
July 3	Water suspension, in 2.5% O_2	5.2	2.7	1.9	2.1	1.7	
	Water suspension, in 1% O_2	6.0	2.7	2.1	2.2	1.7	
	0.2% glucose suspension, in air	7.2	5.2	4.5	4.4	3.7	
	0.2% glucose suspension, in 2.5% O_2	11.0	5.1	4.0	3.7	3.1	

TABLE II—*Continued*

Date 1929	Condition	Q_{O_2} of *Sarcina lutea* Hours after start of experiment					
		1	2	3	4	5	6
Aug. 7	Water suspension, in air	8.6		←— 4.1 —→			
	Water suspension, in air after 4 hours anoxia	14.4		6.2			
	Water suspension, in air after 4 hours anoxia	11.8		6.0			
	0.2% glucose suspension, in air	21.1		11.6			
	0.2% glucose suspension, in air after 4 hours anoxia	34.8		19.0			
	0.2% glucose suspension, in air after 4 hours anoxia	31.8		16.6			
Oct. 16	Water suspension, in air	3.2	2.1	1.8		←—1.2—→	1.1 (9 hrs.)
	Water suspension, in air after 1 hour N_2 bubbling	3.3	←—	1.7 —→		←—	1.1 —→ (9 hrs.)
	Water suspension, in air after 1 hour O_2 bubbling	1.8	←—	1.4 —→		←—	1.1 —→ (9 hrs.)
	Water suspension, in air after 13 hours anoxia	9.1		2.1			
	0.2% glucose suspension, in air after 1 hour anoxia	7.5	←—	3.8 —→		←—	2.1 —→ (9 hrs).
	0.2% glucose suspension, in air after 13 hours anoxia	12.2		4.6			
Nov. 14	Water suspension, in air	4.3	←—3.1—→		←—1.7—→		←—1.3—→ (9 hrs.)
	Same	4.3	←—2.5—→		←—1.7—→		←—1.3—→ (9 hrs.)
	0.2% glucose suspension, in air	9.3	←—3.3—→		←—1.8—→		←—1.6—→ (9 hrs.)
	Same	8.8	←—4.5—→		←—2.0—→		←—1.6—→ (9 hrs.)

excluded as factors leading to the fall in oxygen consumption. It might then be assumed that the initial values are excessive, probably largely as a result of partial asphyxia of the organisms induced in the course of their preparation (and partly perhaps due to food reserves stored from the nutrient medium), and that the lower values obtained some time after an experiment is begun are to be regarded as the "normal" ones rather than as "abnormal" levels resulting from some depression. It may be noted that Callow (1924) obtained a Q_{O_2} of 7.0, constant for 10 hours, using *Sarcina aurantiaca*.

When the organisms are suspended in a 0.2–0.5 per cent glucose solution instead of water, the same progressive fall of oxygen consumption is observed. The Q_{O_2} values at each point in time are, however, much greater in the former case (by 100 per cent to 300 per cent), so that the respiration curve for the bacteria in the glucose-containing menstruum lies above but parallel to that for the bacteria in water. (Fig. 1, Table II.) The influences of glucose and other substances on the respiration of *Sarcina lutea* are reported upon in more detail in the following paper.

The R.Q. in water and in glucose solution was determined in two experiments. In water it remained constant at about 0.67 during 8 hours. (Gotschlich, 1912, cited quotients of 0.71–0.78 for bacteria metabolizing in the absence of fermentable substances, and Stephenson and Whetham, 1923, found R.Q.'s of about 0.7 after all added glucose had been utilized.) In glucose solution, the R.Q. for the first hour was 0.95 and then fell during seven hours to 0.71, the average for the whole run being 0.81. These figures suggest that, even in glucose solution, less than half the oxygen consumed is used to oxidize carbohydrate, although the presence of glucose has more than doubled the amount of oxygen taken up. (These values of R.Q. are lower than those recorded for bacteria on a nutrient pabulum capable of supporting their growth. *Vide* Soule, 1928.)

2. *The Influence of Oxygen Pressure.*—The oxygen consumption of *Sarcina lutea* in equilibrium with oxygen at varying partial pressures has been determined and it has been found that in air or pure oxygen the respiration is essentially the same. When the bacteria were held in water, with a lower rate of respiration, 2.5 per cent oxygen in the gas or even one per cent oxygen, in two cases, sufficed as well as the pure gas. With the glucose menstruum (and more rapid oxygen consumption) one per cent oxygen was definitely too low a concentration to support full respiration and 2.5 per cent of the gas was sometimes inadequate. Concentrations below one per cent were uniformly insufficient to support the rate of oxygen consumption possible in air.

When, after a period of complete anoxia, oxygen was admitted to a concentration of 0.5 per cent or less, there was at once a moderate oxygen consumption. Although the rate of respiration was already far less than in air, it fell off considerably in a short time, much as previously described for experiments in air (Fig. 1). The fall in this case was not due to a diminishing supply of oxygen following utilization of the small amount admitted, for it occurred when only 5 to 10 per cent of the oxygen had disappeared. More than doubling the amount of shaking did not affect the value of the critical oxygen pressure That failure to attain equilibrium between gas and liquid phases did not play a rôle is further attested by experiments made in another connection. Hydrogen was absorbed by an unsaturated fat in water suspension under the influence of a catalyst at a rate over 100 times that of the oxygen absorption here, yet the rate of shaking was adequate.

3. *Oxygen Debt.*—It seemed possible that the regular occurrence of a relatively high oxygen consumption directly after admitting this gas, or at the start of an experiment as the bacterial suspensions became well oxygenated by shaking, might represent the discharge of an oxygen debt. That such a debt can be accumulated is easily demonstrated: a suspension kept for some hours in nitrogen and

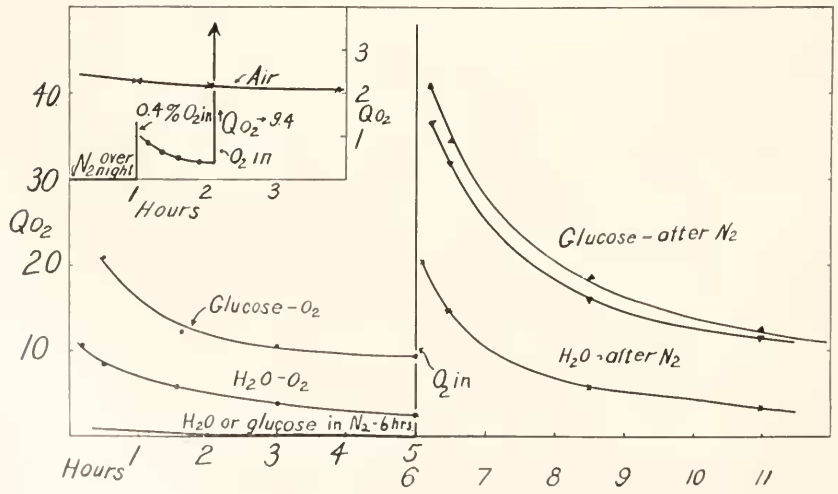

Fig. 2.

then exposed to air takes up oxygen at a greatly increased rate (Fig. 2). At the start, this rate may be considerably greater than observed when the bacteria are kept in air throughout. It subsequently

decreases just as under the conditions previously considered. This burst of increased oxygen consumption is greater after long than after short periods of anoxia, and for the same period is greater when the bacteria are suspended in glucose solution than in water (Table II). Glucose is capable, apparently, of stimulating the metabolism of these organisms not only in the presence of oxygen (leading to a more rapid consumption of the gas), but also in its absence (leading to an increased oxygen debt).

To determine whether previous anoxia was the cause of the high respiration observed at the start of the simple experiment in air, the following test was made. A particular bacterial suspension was divided into two portions. Oxygen was bubbled through one and nitrogen through the other for an hour and then each was rapidly introduced into manometers in air. The usual high initial oxygen consumption values were obtained with the sample treated with nitrogen, while they were much lower for the oxygen-treated portion. The slow fall, continuing for many hours after mounting, occurred with both. This slow fall of oxygen consumption with time might be related to the exhaustion of the substrate to be oxidized rather than to the oxygen itself. In the case of water suspensions, at least, the only material for oxidation that the cells have is their own substance and such food as may be stored during growth in a nutrient medium. During 24 hours the carbon in the carbon dioxide given off corresponds to 1 to 2 per cent of the dry weight of the organisms.

4. *Acid Formation.*—The oxygen debt these cells incur in an atmosphere of nitrogen might be due to accumulation of oxidizable metabolites or to exhaustion of an oxidizing reserve. The first case is reminiscent of the metabolism of muscle and the rôle played by lactic acid, the second suggests the metabolism of nerve. We therefore attempted to ascertain whether acid is produced in the metabolism of the microbic cells under the conditions of our experiments—especially in the absence of oxygen.

The pH of the cell suspension was determined in many experiments before and after manipulation in the manometers, using the indicator method described above. There was no consistent change under any of the conditions studied—water or glucose solution, oxygen present or absent. The initial and final pH values were about 7.4, though occasional final values were as high as 8.0 and as low as 6.0, especially in glucose suspensions. Acid formation was also measured manometrically by the liberation of CO_2 from bicarbonate-Ringer solution in equilibrium with 5 per cent CO_2 in N_2. This was studied in the presence or absence of glucose. During eight hours' asphyxia, the

CO_2 liberated, if due to lactic acid formation, indicated a production of 0.0023 mgm. of lactic acid per mgm. dry weight per hour in glucose solution and 0.0001 mgm. lactic acid in water (one experiment each). The quantities of lactic acid formed in eight hours would require, respectively, 14.5 and 0.6 cu. mm. of O_2 for complete oxidation. For many cells, including bacteria, studied by Meyerhof (1927) (also: Meyerhof and Finkle, 1925) and others, the oxidation quotient (the lactic acid produced in nitrogen minus that formed in oxygen, divided by the oxygen consumed) is 3 or more. That is, one equivalent of oxygen consumed prevents the appearance of three times as many equivalents of lactic acid as it could oxidize. In these experiments, in eight hours' exposure in an oxygen atmosphere, the bacteria consumed: in glucose solution, 24 cu. mm., and in water, 17.5 cu. mm., of oxygen. The oxidation quotients are, therefore, 0.6 and 0.03. The lactic acid (?) produced during asphyxia was only one-fifth of what might have been anticipated in the glucose solution and was practically zero in the water.

These results may be expressed in another way. In other experiments, in either water or glucose, following a six-hour asphyxia the average extra oxygen taken up during 6 hours was 50 per cent of the normal oxygen consumed by the same number of bacteria in the same time. The total oxygen debt in these experiments may therefore be assumed to have been 9 cu. mm. in glucose solution and 5 cu. mm. in water. Since in the water suspension only 0.6 cu. mm. of oxygen could have been used to oxidize acid metabolites (typified by lactic acid), nine-tenths or more must have acted in some other manner. In glucose solution, the extra oxygen might have been used entirely to oxidize metabolites, though presumably here also some would be used otherwise. The possibility must be recognized, of course, that more acid may have been formed than is recorded by the CO_2 liberation, some being neutralized by other cell buffers. Also, intermediate substances of too weak acidity to result in the liberation of CO_2 would escape detection by the methods used.

5. *Oxidizing Reserve.*—The existence of a large oxygen debt after asphyxiation, with no demonstrable accumulation of incompletely oxidized metabolites to account for it, suggests that the extra oxygen is utilized to replenish an oxidizing reserve which had been drawn upon to continue oxidations during asphyxia. If this were the case, some CO_2 should appear during the asphyxial period and a correspondingly smaller amount during the following oxygen period. The R.Q. of the extra respiration, while the oxygen store is being refilled, should be correspondingly low. This does not appear to be the case,

for the R.Q. of suspensions in water is the same early or later after commencing a series of observations, whether or not the extra oxygen consumption is occurring. Also, the absence of CO_2 liberation in nitrogen from water-bicarbonate suspensions renders improbable the production of CO_2 as well as of non-volatile acids. We do not wish, on the basis of the few experiments performed, to conclude finally that the oxygen debt developed by *Sarcina lutea* is due to the accumulation of non-acid metabolites and not to the depletion of oxidizing reserves, though this seems most probable. The work unfortunately could not be continued at the time and the present report must suffice to record these preliminary observations. It is interesting to note that Meyerhof (1912) found in some of his studies on *Vibrio metchnikovi* that in the absence of oxygen all chemical activity and energy liberation appeared to be simply suspended. The falling Q_{O_2} with time observed for *Sarcina*, in contrast to the constant respiration of bacteria observed by Callow (1924), may be related to such differences.

Summary

The respiration of a strain of *Sarcina lutea*, growing as individual cocci of uniform size and of relatively high viability under our experimental conditions, was studied by means of the Warburg technique. Suspensions of thoroughly washed bacteria in water or in glucose solution were enumerated by direct and plate counts, and pH determinations were made before and after manipulation in the manometer. A simple method is described for obtaining accurate gas mixtures with a low concentration of oxygen.

The oxygen consumption of various suspensions of the washed cocci was found to be fairly constant in water suspension at about 2.6 cu. mm. O_2 per mgm. dry weight per hour, or approximately 7 μ^3 O_2 per single cell per hour: that is, over three times its volume of oxygen was consumed per hour by each micrococcus. This value is for the nearly constant level attained some hours after the start of the experiment. Oxygen consumption is considerably more rapid at first and falls along a roughly arithlogarithmic curve towards an asymptote. This early excess appears to represent an oxygen debt due to partial asphyxia produced in the course of preparation of the suspensions.

In 0.2 to 0.5 per cent glucose solution, the rate of oxygen consumption falls along a similar curve, but the rates are 100 to 300 per cent greater than for suspensions of cocci in water.

The respiratory quotient in water is constant at about 0.67; in glucose it falls from 0.95 to 0.71 in seven hours (one experiment each).

The oxygen consumed by a suspension in water is independent of oxygen concentration when this is above one per cent. For glucose suspensions 2.5 per cent O_2 or more is required for maximal consumption. Below the critical values of oxygen tension, oxygen consumption becomes less with diminishing oxygen concentration. When air is admitted to the suspension after a period of complete anoxia, there is observed a high initial rate of consumption followed by progressively declining rates during several hours until a relatively constant rate is approached. When small amounts of oxygen (0.5 per cent or less) are admitted after anoxia, consumption is greater at first and then falls as when air is admitted; but the maximal rate is low, usually less than the normal in air.

The oxygen debt developed by these organisms is not apparently associated with the production of detectable acid metabolites (in water suspensions, at least), nor with the formation of carbon dioxide during asphyxia. It may result from the accumulation of non-acid metabolites, or of amounts of acid metabolites that do not equal or exceed the buffering capacities of the cells. The oxidation quotient, even in glucose solution, in contrast to the data for most cells that have been studied, is not over 0.6.

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